ISSN 1346-7565 Acta Phytotax. Geobot. 57 (3): 205–217 (2006)

Phylogenetic Relationships and Chromosomal Evolution of Japanese *Fimbristylis* (Cyperaceae) Using nrDNA ITS and ETS 1f Sequence Data

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ITS and ETS 1f sequence data were used to estimate the phylogeny of 19 taxa of Japanese *Fimbristylis*. This data was supplemented with karyomorphological observations of 15 taxa within this genus. Molecular analyses indicated strong support for monophyly of the genus *Fimbristylis* including *F. ovata* (= *Abildgaardia ovata*). Our results show that the sections *Dichelostylis* sensu Ohwi (1944), *Cymosae* (excluding *F. pierotii*), and *Trichelostylis* sensu Kern (1974) are monophyletic, but that the sections *Trichelostylis* sensu Ohwi (1944), *Fimbristylis* and *Dichelostylis* sensu Kern (1974) are not. Three major groups were identified in the genus *Fimbristylis* by karyomorphological data: (1) $2n = 10$, 20 and 30, and large somatic chromosomes, (2) $2n = 16$ and 24, and karyotypes consisting of a bimodal arrangement of somatic chromosomes, and (3) $2n = 42$, 44 and 52, and very small somatic chromosomes. The ancestor of the genus *Fimbristylis* is thought to have had a basic chromosome number of $x = 5$ and large chromosomes. Chromosomal evolution in the genus *Fimbristylis* with diffuse centromeric chromosomes may have been affected by both aneuploidization and polyploidization.

Key words: chromosomal evolution, Cyperaceae, diffuse centromeric chromosome, ETS, *Fimbristylis*, ITS, molecular phylogeny

The genus *Fimbristylis* (L.) Vahl includes approximately 100-300 widely distributed species that occur mainly in tropical, subtropical and warm-temperate regions (Kral 1971, 2002, Koyama 1974, Goetghebeur 1998). The genus *Fimbristylis* is closely related to the genera *Abildgaardia*, *Bulbostylis*, *Crosslandia*, *Nelmesia* or *Nemum* (Bruhl 1995, Goetghebeur 1998). However, based on morphology, the genus *Fimbristylis* can be distinguished from other genera by characters such as achene size and ornamentation, embryo form, floral scale form, having inflorescences with many spikelets, and deciduous style-base (Tucker 1987, Goetghebeur 1998).

In the genus *Fimbristylis*, while several intrageneric classifications have been proposed, most of them were based on specimens from a restricted geographic region (Ohwi 1938, 1944, Koyama 1961, 1974, Kern 1974). In Japanese *Fimbristylis*, Ohwi (1938) recognized four subgenera and thirteen sections. Later, Ohwi (1944) recognized thirty-seven species within the Asian *Fimbristylis*, and classified them into five sections and eleven series. An intrageneric classification including many Asian *Fimbristylis* was proposed in a monograph by Kern (1974), who recognized eighteen sections within the genus *Fimbristylis* (Fig. 1). Ohwi (1944) and Kern (1974) included the genus *Abildgaardia* within the genus *Fimbristylis*.

Based on a molecular phylogenetic study of the Cyperaceae, Muasya *et al.* (2000) reported that the genus *Fimbristylis* forms a clade with the genera

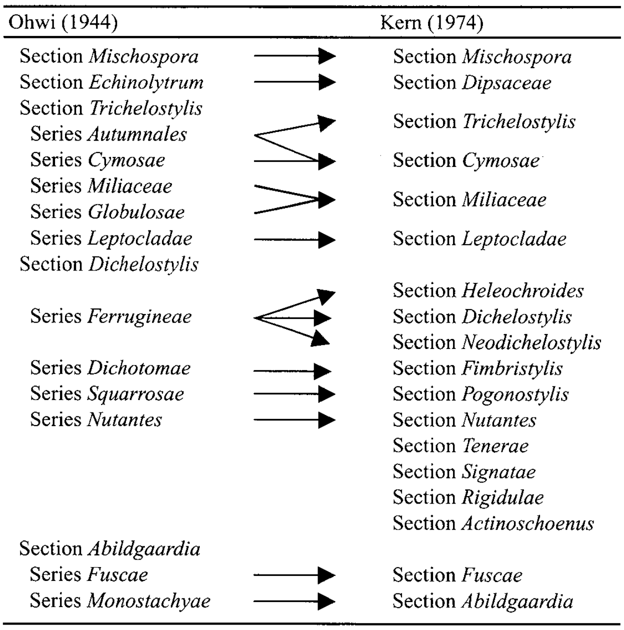

FIG. 1. Comparison of classifications in the genus *Fimbristylis*.

Abildgaardia, *Bulbostylis* and *Nemum*. However, no molecular phylogenetic study has been conducted on the intrageneric relationships of the genus *Fimbristylis*.

The chromosome numbers of the genus *Fimbristylis* have been reported previously by Tanaka (1939), Skottsberg (1955), Kral (1971), Mehra & Sachdeva (1975), Rath & Patnaik (1978), Probatova & Sokolovskaya (1981), Bir *et al.* (1988, 1992) and Yano *et al.* (2006). The lowest chromosome number in the genus was $2n = 10$ (Tanaka 1939) and the highest was $2n = 60$ (Kral 1971). Cytological studies on Japanese *Fimbristylis* were conducted by Tanaka (1939) and Yano *et al.* (2006) who observed chromosome numbers of $2n = 10$, 16, 20, 24, 30 and 44 in twelve taxa. Intraspecific polyploidy has frequently been reported within the genus (Kral 1971, Mehra & Sachdeva 1975, Bir *et al.* 1988, 1992), but no studies have been conducted on the relationship between chromosomal evolution and phylogeny.

DNA sequences are commonly employed in phylogenetic analyses and have been applied previously to the Cyperaceae (Muasya *et al.* 1998, 2000, Simpson *et al.* 2003). The nuclear ribosomal internal transcribed spacer (ITS) and external transcribed spacer 1 fragment (ETS 1f) sequence data have been used to estimate lower-level intrageneric relationships (Starr *et al.* 2003, 2004, Roalson & Friar 2004, Yano & Hoshino 2005). Our study aims to clarify the intrageneric relationships of the Japanese *Fimbristylis* using nrITS and nrETS 1f sequence data of almost all Japanese *Fimbristylis*, and also to examine the relationship between chromosomal evolution and molecular phylogeny.

Materials and Methods

Plant samples

Nineteen taxa of Japanese *Fimbristylis* were sampled for phylogenetic analysis (Table 1). In this study, *Abildgaardia ovata* was treated as *Fimbristylis ovata* and was regarded as a member of the genus *Fimbristylis*. To test for monophyly of the genus *Fimbristylis*, we selected *Eleocharis atropurpurea*, *E. congesta* var. *japonica* and *Trichophorum alpinum* as outgroups.

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from 0.1 g of fresh-frozen material or 0.03 g of dried specimens using a Nucleon Phytopure plant and fungal DNA extraction kit (Amersham Inc.). Amplification of the nrDNA internal transcribed spacers (ITS) region and the nrDNA noncoding fragments from the external transcribed spacers 1 (ETS 1f) region were performed by polymerase chain reaction (PCR) with Taq polymerase (TaKaRa Inc.). Reactions were performed using a DNA thermal cycler (Program Temp Control System PC-320, Astec Inc.) following the protocol of Hsiao *et al.* (1994) to amplify the ITS region, and the protocol of Starr *et al.* (2003) for amplifying the ETS 1f region. The ITS region was amplified using the ITSL and ITS4 primers designed by Hsiao *et al.* (1994) and White *et al.* (1990), and the ETS 1f region was amplified using the ETS 1f and 18S-R primers designed by Starr *et al.* (2003). Additional internal primers, ETS1fA (5'-TTCATG-GCGGGCTCCCTG-3'; forward) were also used for the amplification of the ETS 1f region. PCR products were electrophoresed on 1.4% agarose gels to confirm the presence of a single product and purified using a Qiaquick PCR purification Kit (Qiagen Inc.).

Cycle sequencing reactions were performed for double strands using the purified PCR products and a BigDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems Inc.). ITS and ETS 1f sequencing used the same primers that were employed for the respective amplification reactions. The products were resolved by capillary electrophoresis using an ABI PRISM 310 automated DNA sequencer (Applied Biosystems Inc.). Sequences were assembled and edited using Sequencing Analysis version 3.0 (Perkin Elmer Inc.).

Data analysis

The data matrices for the ITS, ETS 1f and ITS+ETS 1f sequences, including the nineteen taxa of Japanese *Fimbristylis*, one *Bulbostylis* and three outgroups, were used for the phylogenetic analyses. Sequences were aligned using Clustal W (Thompson *et al.* 1994) and adjusted manually as necessary. Phylogenetic analyses were performed following the methods of Yano *et al.* (2004) and Yano & Hoshino (2005). Phylogenetic trees were constructed using the methods of maximum-parsimony (MP) (Fitch 1971) and maximum-likelihood (ML) (Felsenstein 1981) for each region. The HKY85 model (Hasegawa *et al.* 1985) was used as the estimate model for ML analyses. A transition / transversion (Ts / Tv) parameter of 2.00/2.03/2.07 (ITS / ETS 1f / ITS+ETS 1f) was used based on the derived using PUZZLE version 5.0 (Strimmer & Haeseler 1996) for ML analyses.

Tree comparisons using ML criteria were performed using NucML of MOLPHY version 2.3b3 (Adachi & Hasegawa 1996) to evaluate the topology of trees obtained from the two methods (ML and MP). CONSEL 0.1f (Shimodaira & Hasegawa 2001) was used to calculate the *p*-values for estimating confidence levels of bifurcating candidate topologies using test procedures. The highest log-likelihood ITS tree, ETS 1f tree and ITS+ETS 1f tree were obtained using TotalML of MOLPHY to evaluate the resulting trees.

For the combined ITS and ETS 1f dataset, MP trees were also constructed using MEGA version 2.1 (MEGA2; Kumar *et al.* 2001). In analyses with MEGA2, MP trees were estimated using the Close-neighbor-interchange (CNI) method with a search level of 3. The CNI search was repeated 10 times, starting with trees generated after 1,000 replications with random addition sequences. Support for each clade was assessed using 1,000 bootstrap replicates (BS; %; Felsenstein 1985).

Chromosome observations

Fifteen taxa within the genus *Fimbristylis* were collected in Japan and subjected to karyomorphological analysis, voucher specimens for which are listed in Table 1. Somatic chromosomes of the collected taxa were observed in meristematic cells of root tips pretreated with 0.002 M 8-hydroxyquinoline for 5 h at 16°C or for 1 h at 23°C and 15 h at 4°C. These roots were then fixed in acetic alcohol (1: 3) for over 16 h at -20°C or for 1.5 h at 23°C, stained by Feulgen's nuclear reaction, macerated in a mixture of 2% pectinase and 2% cellulase for 1 h at 37°C, restained in 1% aceto-orcein, and squashed. Meiotic chromosomes were also observed in pollen mother cells (PMCs). Spikelets were fixed in acetic alcohol (1: 3) for over 6 h at -20°C. The anthers were stained in 1% aceto-orcein and then squashed.

Results

Phylogenetic analyses

TABLE 1. Taxa used in this study. Genbank accession numbers listed for ITS and ETS 1f data. Vouchers are deposited in OKAY.

Taxon Locality and Voucher	Chromosome number; 2n (n)	Previous report (2n)	Accession number ITS	ETS 1f
***Fimbristylis* Vahl**				
F. aestivalis (Retz.) Vahl		10 (Tanaka 1939)		
Japan, Nagano Pref., Kita-saku; *Arai &Hirahara 18166* (OKAY)			AB250626	AB250645
F. autumnalis (L.) Roem. et Schult.		10 (Tanaka 1939)		
Japan, Toyama Pref., Toyama; *Yano 20499* (OKAY)	10		AB250627	AB250646
F. complanata (Retz.) Link		16 (Tanaka 1939)		
Japan, Okayama Pref., Okayama; *Yano 20422* (OKAY)	16		AB250628	AB250647
F. cymosa R.Br.		28, 48 (Skottsberg 1955)		
Japan, Kagoshima Pref., Mishima; *Hoshino et al. 19704* (OKAY)	52		AB250629	AB250648
Japan, Okinawa Pref., Nago; *Tsusaka 20562* (OKAY)	52			
F. dichotoma (L.) Vahl f. *annua* (All.) Ohwi		10, 20, 30 (Tanaka 1939, Kral 1971, Mehara & Sachdeva 1975, Bir *et al.* 1988, 1992)		
Japan, Okayama Pref., Sohjya; *Katayama 17519* (OKAY)			AB250630	AB250649
Japan, Hiroshima Pref., Yamagata; *Fujii 20459* (OKAY)	20			
F. dichotoma (L.) Vahl f. floribunda (Miq.) Ohwi				
Japan, Okinawa Pref., Ishigaki; *Ikeda et al. 19723* (OKAY)	30 (15II)		AB250631	AB250650
Japan, Okinawa Pref., Yaeyama; *Hirahara 20352* (OKAY)	30			
F. diphylloides Makino		20 (Tanaka 1939)		
Japan, Okayama Pref., Okayama; *Yano 20517* (OKAY)	10		AB250632	AB250651
F. ferruginea (L.) Vahl var. *anpinensis* (Hayata) H.Y.Liu				
Japan, Okinawa Pref., Yaeyama; *Hirahara & Yano 19326* (OKAY)			AB250633	AB250652
Japan, Okinawa Pref., Yaeyama; *Hirahara 20350* (OKAY)	10			
F. ferruginea (L.) Vahl var. *sieboldii* (Miq. ex Franch. et Sav.) Ohwi				
Japan, Okayama Pref., Asaguchi; *Hoshino & Masaki 18254* (OKAY)			AB250634	AB250653
Japan, Kagoshima Pref., Kumage; *Ikeda et al. 20152* (OKAY)	10			
Japan, Kanagawa Pref., Miura; *Yano 20482* (OKAY)	10			
F. longispica Steud. var. boninensis Ohwi		30 (Yano *et al.* 2006)		
Japan, Tokyo Pref., Ogasawara; *Katsuyama et al. 19920* (OKAY)			AB250635	AB250654
F. longispica Steud. var. hahajimensis (Tuyama) Ohwi		30 (Yano *et al.* 2006)		
Japan, Tokyo Pref., Ogasawara; *Katsuyama et al. 19910* (OKAY)			AB250636	AB250655
F. miliacea (L.) Vahl		10 (Tanaka 1939, Kral 1971, Mehara & Sachdeva 1975, Bir *et al.* 1988, 1992)		
Japan, Okayama Pref., Okayama; *Yano 20423* (OKAY)	10		AB250637	AB250656

TABLE 1. continued.

Taxon Locality and Voucher	Chromosome number; 2n (n)	Previous report (2n)	Accession number	
			ITS	ETS 1f
F. ovata (Burm. fil.) Kern		10, 20 (Kral 1971, Bir *et al.* 1992)		
Japan, Kanagawa Pref., Miura; *Yano 18218* (OKAY)			AB180719	AB206286
Japan, Kanagawa Pref., Miura; *Yano 20481* (OKAY)	20			
Japan, Okinawa Pref., Yaeyama; *Tsusaka 19707* (OKAY)	20			
Japan, Okinawa Pref., Ishigaki; *Tsusaka 19708* (OKAY)	20		AB250638	AB250657
F. pierotii Miq.				
Japan, Yamaguchi Pref., Mine; *Hoshino et al. 20053* (OKAY)	42		AB250639	AB250658
F. sericea (Poir.) R.Br.		44 (Tanaka 1939)		
Japan, Tottori Pref., Tottori; *Yano 20491* (OKAY)	44		AB250640	AB250659
Japan, Mie Pref., Age; *Tsusaka 20495* (OKAY)	44			
F. squarrosa Vahl		20 (Tanaka 1939, Mehara & Sachdeva 1975)		
Japan, Nagano Pref., Kita-saku; *Arai & Hirahara 18165* (OKAY)			AB250641	AB250660
F. subbispicata Nees et Meyen		10 (Tanaka 1939)		
Japan, Okayama Pref., Sohjya; *Katayama 17512* (OKAY)			AB250642	AB250661
Japan, Okayama Pref., Okayama; *Yano 19628* (OKAY)	10			
F. velata R.Br.		24 (Tanaka 1939)		
Japan, Okayama Pref., Okayama; *Katayama 18206* (OKAY)			AB250643	AB250662
Japan, Tottori Pref., Tottori; *Yano 20492* (OKAY)	24			
Japan, Tottori Pref., Tottori; *Yano 20494* (OKAY)	24			
F. verrucifera (Maxim.) Makino		10 (Probatova & Sokolovskaya 1981)		
Japan, Okayama Pref., Okayama; *Katayama 18208* (OKAY)			AB250644	AB250663
Japan, Okayama Pref., Okayama; *Yano 20515* (OKAY)	10			
***Bulbostylis* Kunth**				
B. barbata Kunth				
Japan, Okayama Pref., Okayama; *Hoshino et al. 13779* (OKAY)			AB180718	AB206285
***Eleocharis* R.Br.**				
E. atropurpurea (Retz.) Presl				
Japan, Okayama Pref., Okayama; *Hoshino et al. 18311* (OKAY)			AB180694	AB250664
E. congesta D. Don var. *japonica* (Miq.) T. Koyama				
Japan, Okayama Pref., Okayama; *Hirahara & Yano 18128* (OKAY)			AB180697	AB250665
***Trichophorum* Pers.**				
T. alpinum (L) Pers.				
Japan, Hokkaido Pref., Nakagawa; *Sato 13260* (OKAY)			AB206270	AB206287

The ITS sequences of the 23 taxa, including the three outgroups, ranged in length from 445 to 465 bp. Aligned sequences measured 516 bp, of which 403 sites were used for phylogenetic analysis. The data matrix contained a total of 243 variable sites, of which 165 were potentially phylogenetically informative. The ETS 1f sequences ranged in length from 506 to 616 bp. Aligned sequences measured 658 bp, of which 426 sites were used for phylogenetic analysis. The data matrix contained 298 variable sites, of which 191 were potentially phylogenetically informative. Gaps were treated as missing data and not used as characters. For the combined ITS and ETS 1f dataset, 829 sites were included, of which 541 were variable and 356 were potentially phylogenetically informative.

For phylogenetic analyses, a total of four (ITS), three (ETS 1f) and two (ITS+ETS 1f) non-overlapping topologies were obtained using the MP and ML methods. More detailed topologies were searched for among the obtained trees using the log-likelihood measure (NucML and CONSEL 0.1f). The highest log-likelihood tree for the ITS, ETS 1f, and ITS+ETS 1f was obtained and is shown in Fig. 2. For the combined ITS and ETS 1f dataset, two equally most parsimonious topologies were obtained using the MP method. The bootstrap consensus tree (> 50%) of the MP tree is shown in Fig. 3.

Phylogenetic analysis of the Japanese species strongly supported the monophyly of the genus *Fimbristylis* including *F. ovata* (= *Abildgaardia ovata*), with 100/99% support for the Total ML tree and a 100% BS value for the MP tree. Genus *Bulbostylis* was sister to *Fimbristylis* clade in ML and MP trees. In the genus *Fimbristylis*, the *F. subbispicata* / *F. aestivalis* clade formed a monophyletic group with strong support (93/100% support in the Total ML tree and a 99% BS value in the MP tree). However, the relationships between *F. diphylloides*, *F. verrucifera*, *F. pierotii* and *F. sericea* / *F. cymosa* were not resolved. Instead, these taxa exhibited polytomy in the MP tree (Fig. 3) and their relationships were weakly supported in the Total ML tree (Fig. 2).

Chromosome observations

Cytological studies were conducted in 15 taxa. Chromosome numbers were observed to be $2n = 10$ for *Fimbristylis autumnalis*, $2n = 10$ for *F. miliacea*, $2n = 10$ for *F. subbispicata*, $2n = 10$ for *F. verrucifera*, $2n = 16$ for *F. complanata*, $2n = 20$ for *F. dichotoma* f. *annua*, $2n = 20$ for *F. ovata*, $2n = 24$ for *F. velata* and $2n = 44$ for *F. sericea*, corroborating previous reports (Tanaka 1939, Kral 1971, Mehra & Sachdeva 1975, Probatova & Sokolovskaya 1981, and Bir *et al* 1988, 1992) (Table 1). The chromosome numbers of $2n = 10$ for *F. ferruginea* var. *anpinensis*, $2n = 10$ for *F. ferruginea* var. *sieboldii*, $2n = 30 = 15\text{II}$ for *F. dichotoma* f. *floribunda* and $2n = 42$ for *F. pierotii* were determined for the first time in this study. Chromosome numbers determined in this study that differed from those that had been published previously $2n = 10$ for *F. diphylloides* and $2n = 52$ for *F. cymosa* (Fig. 4, Table 1).

The genus *Fimbristylis* can be classified into the following three major groups based on the karyotype analysis (Fig. 4): (1) $2n = 10$, 20 and 30, and had a basic chromosome number $x = 5$. Somatic chromosomes of these taxa exhibited a gradient with respect to length, ranging from 1.6 μm to 3.1 μm in length (Fig. 4A-G, I, J, L). (2) $2n = 16$ and 24, that had a bimodal karyotypic distribution with respect to length. The $2n = 16$ had 4 large chromosomes that ranged from 3.9 μm to 4.4 μm and the other 12 chromosomes ranged from 1.9 μm to 2.8 μm in length (Fig. 4H). The $2n = 24$ had 12 small chromosomes that ranged from 0.9 μm to 1.3 μm and the other 12 chromosomes ranged from 1.6 μm to 1.9 μm in length (Fig. 4K). (3) $2n = 42$, 44 and 52 had small chromosomes. The somatic chromosomes of these taxa exhibited a gradient with respect to size and were less than 1.2 μm in length (Fig. 4M-O).

Discussion

Inter and intrageneric relationships

Muasya *et al.* (2000) reported the following two topologies in the phylogenetic relationships of the tribe *Abildgaardieae*, based on *rbcL* sequence data. (1) genus *Fimbristylis* is closely related to genus *Abildgaardia*, and *Fimbristylis* / *Abildgaardia* clade is sister to *Bulbostylis* / *Nemum* clade. (2) genus *Fimbristylis* is closely related to *Bulbostylis* / *Nemum* clade, and *Fimbristylis* / *Bulbostylis* / *Nemum* clade is sister to genus *Abildgaardia*. In our study, the genera *Fimbristylis* and *Abildgaardia* formed a monophyletic group with strong support

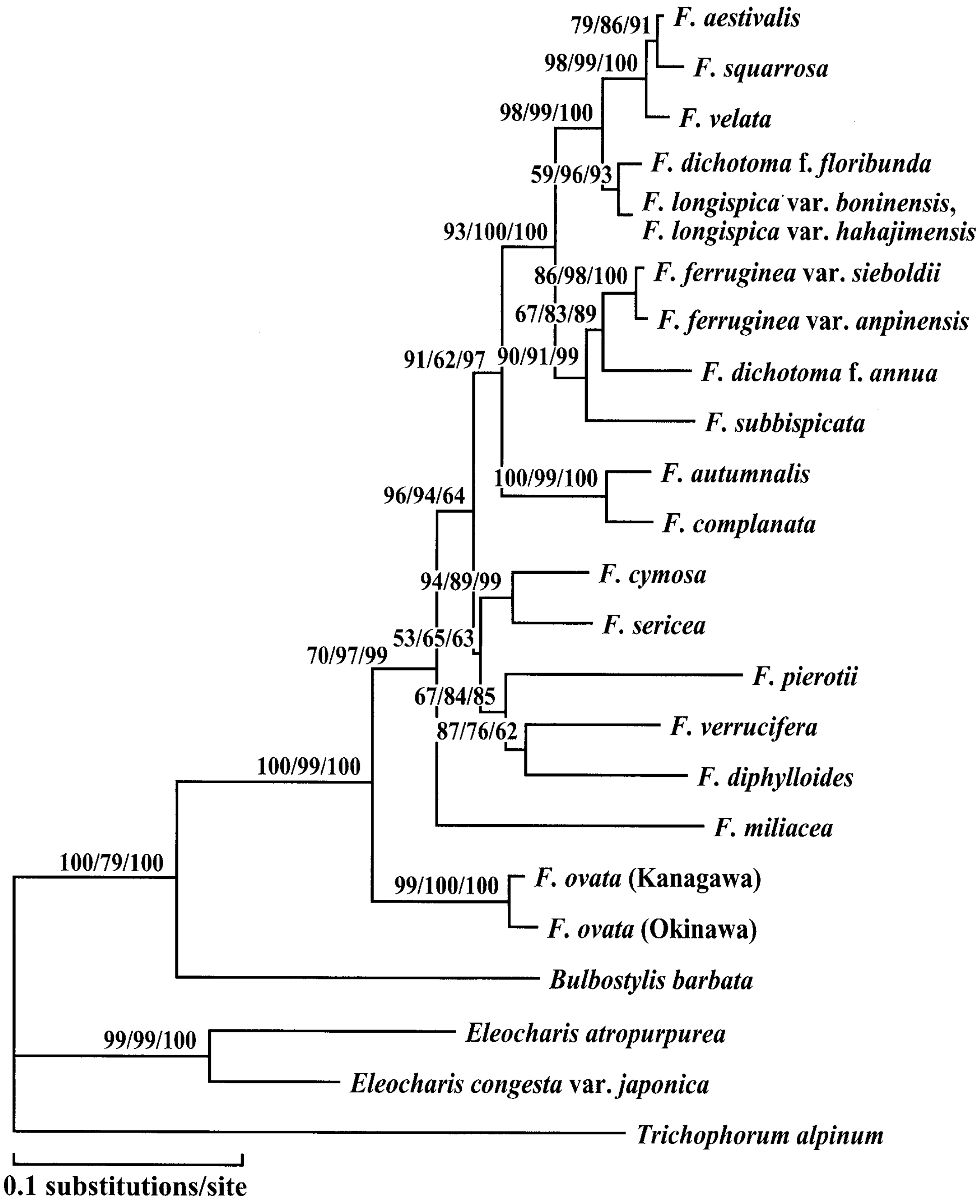

FIG. 2. Total ML tree of the ITS and ETS 1f sequences in Japanese *Fimbristylis* (HKY85 model, ln L=-13860.5 by NucML). Local bootstrap probabilities (LBP; %) more than 50% are shown above branches (ITS/ETS 1f/ITS+ETS 1f).

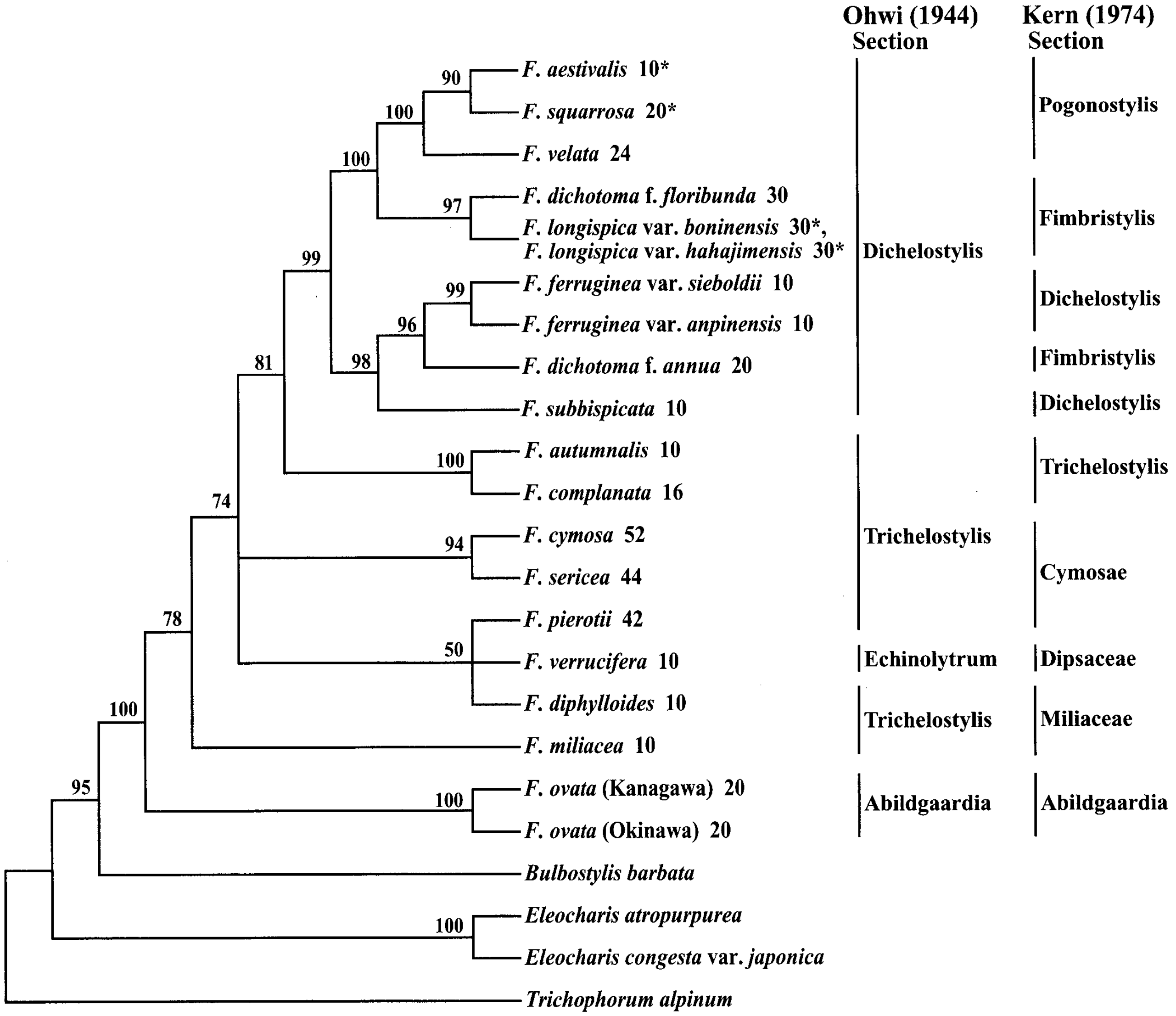

FIG. 3. Bootstrap consensus tree (>50%) of the two equally most parsimonious trees of combined ITS and ETS 1f data set in Japanese *Fimbristylis* (Tree length=1268, CI=0.65, RI=0.64). Bootstrap values (BS; %) are shown above branches. The numbers after each species are chromosome numbers (2*n*), and asterisk shows the count by other authors in previous studies. The intrageneric classifications by Ohwi (1944) and Kern (1974) are shown on the right.

(100/99% LBP and 100% BP), and *Fimbristylis* / *Abildgaardia* clade is strongly supported as a sister to the genus *Bulbostylis* (100/79% LBP and 95 % BP). Our results show that genus *Fimbristylis* is most closely related to genus *Abildgaardia*.

Ohwi (1944) recognized five sections, *Abildgaardia*, *Dichelostylis*, *Echinolytrum*, *Mischospora* and *Trichelostylis*, in the genus *Fimbristylis*. In Ohwi's classification, achene form is considered an important character for distinguishing the sections *Echinolytrum* and *Mischospora* from the other three sections. The achenes of the sections *Echinolytrum* and *Mischospora* were oblong-cylindrical, but the achenes of the other three sections were obovoid. However, phylogenetic placement of the section *Echinolytrum* was not resolved in our study and section *Mischospora* could not be included in this study due to the unavailability of plant material. The arrangement of floral scale is considered the major character for distinguishing the section

Abildgaardia from the sections *Dichelostylis* and *Trichelostylis*. The floral scale of section *Abildgaardia* is distichously arranged, but that in sections *Dichelostylis* and *Trichelostylis* it has a spiral arrangement. The style is considered an important character for separating the section *Dichelostylis* from the section *Trichelostylis*. The styles of the section *Dichelostylis* were 2-branched, flat and usually ciliate, but the styles of the section *Trichelostylis* were usually 3-branched, subterete or triquetrous and glabrous.

A comparison of the findings of our phylogenetic tree with the intrageneric classification of Ohwi (1944), reveals an agreement with respect to the section *Dichelostylis* as being monophyletic (99% BP), and the section *Trichelostylis* as not being monophyletic (Fig. 3). Our molecular phylogenetic data also show that the style is an important character for separating the section *Dichelostylis* from the section *Trichelostylis*. In the section *Abildgaardia*, only one species of which was included in this study, was moderately separated from the other sections (78% BP). Our molecular phylogenetic data show that the arrangement of the floral scale is also an important character for distinguishing the section *Abildgaardia* from the sections *Dichelostylis* and *Trichelostylis*. We propose that the arrangement of the floral scale and the style may be important morphological characters for the intrageneric classification of the genus *Fimbristylis*.

Kern (1974) recognized eighteen sections in the genus *Fimbristylis*. Kern adopted part of Ohwi's section and raised Ohwi's series to sectional rank (Fig. 1). Kern included part of Ohwi's series *Autumnales* to the section *Cymosae*, and divided Ohwi's series *Ferrugineae* into three sections. Furthermore, Kern recognized Ohwi's series *Miliaceae* and *Globulosae* as the section *Miliaceae*. In this study, Kern's sections *Pogonostylis* and *Trichelostylis* were monophyletic (100% BP, respectively), but the sections *Cymosae*, *Fimbristylis*, *Dichelostylis* and *Miliaceae* were not monophyletic based on our phylogenetic tree (Fig. 3). However, the section *Cymosae* is monophyletic if *F. pierotii* is excluded (94% BP). Our results infer that *F. pierotii* is separated from section *Cymosae*. In this study, *F. dichotoma* were polyphyletic. The taxa of *F. dichotoma* complex are distributed tropical, subtropical and warm-temperate regions, and many intraspecific taxa were reported (Ohwi 1944, Koyama 1961, 1974, Kern 1974). Taxonomic revision may be needed for *F. dichotoma* complex. Section *Abildgaardia*, only consisting of one species in this study, was moderately separated from the other sections (78% BP). *Fimbristylis diphylloides* in section *Miliaceae* was also separated from *F. miliacea* (74% BP).

Our phylogenetic tree supports monophyly of the section *Dichelostylis* of Ohwi (1944). Our tree also supports the monophyly of the sections *Cymosae* (excluding *F. pierotii*) and *Trichelostylis* of Kern (1974). However, the section *Trichelostylis* sensu Ohwi (1944), and sections *Fimbristylis* and *Dichelostylis* sensu Kern (1974) are not supported by our phylogenetic tree. Our data suggest that the sections *Fimbristylis*, *Dichelostylis* and *Pogonostylis* sensu Kern (1974) should be treated as the section *Dichelostylis* sensu Ohwi (1944). The section *Trichelostylis* sensu Ohwi (1944) should be treated as the sections *Miliaceae* (excluding *F. diphylloides*), *Cymosae* (excluding *F. pierotii*) and *Trichelostylis* sensu Kern (1974). Further morphological analyses are needed to more accurately assess the taxonomical relationships between these groups and more samples should be included to increase our understanding of *Fimbristylis* phylogeny.

Karyotype and chromosomal evolution

Chromosomal evolution of the Cyperaceae with diffuse centromeric chromosomes based on a molecular phylogeny was proposed by Roalson *et al.* (2001), Yano *et al.* (2004) and Yano & Hoshino (2005). Roalson *et al.* (2001) speculated that the

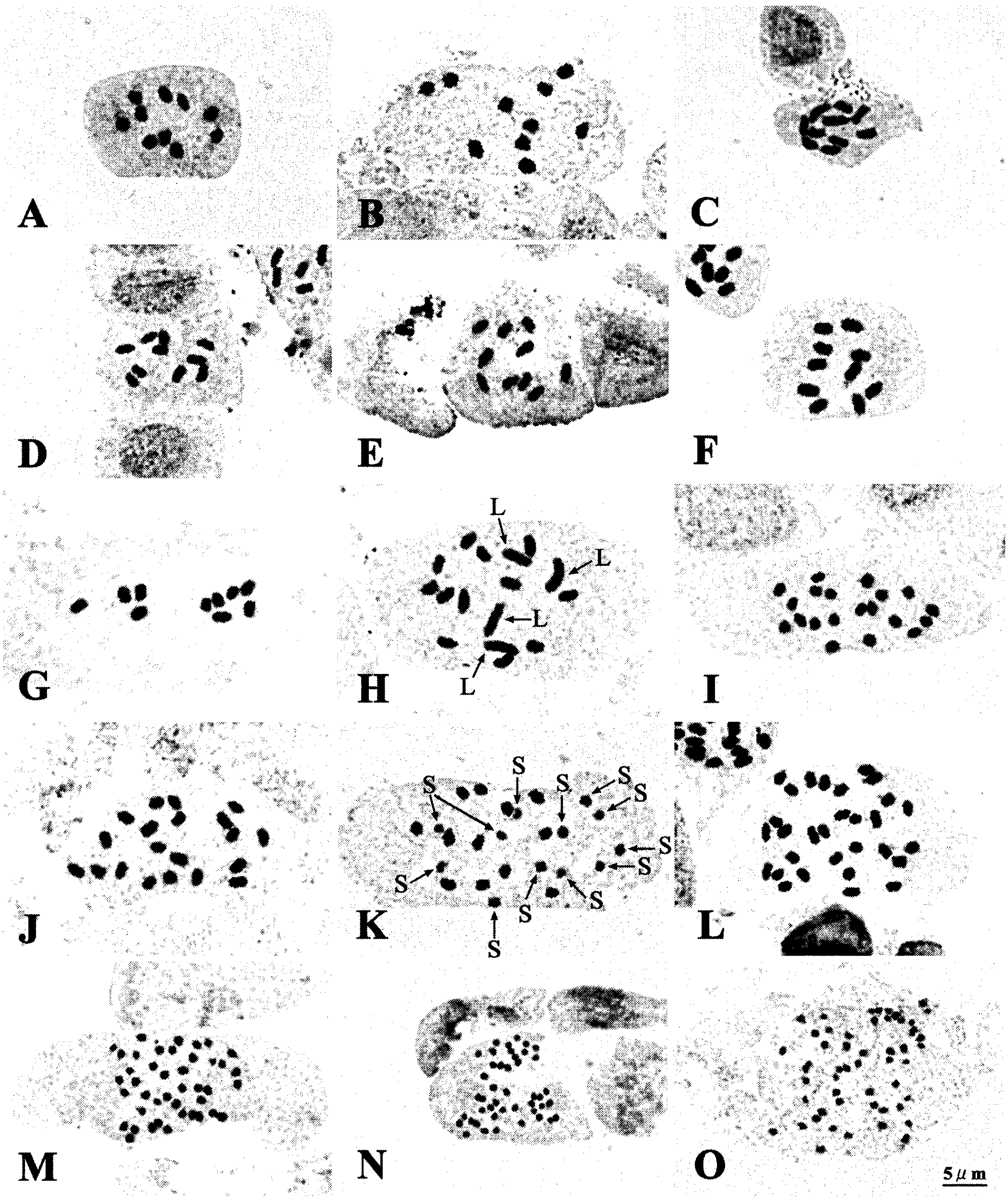

FIG. 4. Photomicrographs of somatic metaphase chromosomes of the genus *Fimbristylis*. **A.** *Fimbristylis autumnalis* (2*n* = 10). **B.** *F. diphylloides* (2*n* = 10). **C.** *F. ferruginea* var. *anpinensis* (2*n* = 10). **D.** *F. ferruginea* var. *sieboldii* (2*n* = 10). **E.** *F. miliacea* (2*n* = 10). **F.** *F. subbispicata* (2*n* = 10). **G.** *F. verrucifera* (2*n* = 10). **H.** *F. complanata* (2*n* = 16). **I.** *F. dichotoma* f. *annua* (2*n* = 20). **J.** *Fimbristylis ovata* (2*n* = 20). **K.** *F. velata* (2*n* = 24). **L.** *F. dichotoma* f. *floribunda* (2*n* = 30). **M.** *F. pierotii* (2*n* = 42). **N.** *F. sericea* (2*n* = 44). **O.** *F. cymosa* (2*n* = 52). Arrows indicate large (L) and small (S) chromosomes.

ancestor of the tribe Cariceae had a moderate to high chromosome number, and that low chromosome numbers might represent a derived condition within the genus *Carex*. Yano *et al.* (2004) attributed chromosome diversity to both aneuploidization and polyploidization in the genus *Eleocharis*. Yano &

Hoshino (2005) reported that chromosomal evolution in the genus *Schoenoplectus* may be caused more by polyploidy than by aneuploidy.

In this study, the genus *Fimbristylis* could be classified into the following three major groups based on karyomorphological observations (Fig. 4): (1) $2n = 10$, 20 and 30, that had large somatic chromosomes (Fig. 4A-G, I, J, L), (2) $2n = 16$ and 24, that had somatic chromosomes exhibiting a bimodal distribution with respect to length (Fig. 4H and K), and (3) $2n = 42$, 44 and 52, and very small somatic chromosomes (Fig. 4M-O). When these karyomorphological data and previously reported chromosome numbers are superimposed on the phylogenetic tree derived in this study (Fig. 3), the ancestor of the genus *Fimbristylis* is postulated to have a basic chromosome number of $x = 5$ and large chromosomes.

Tanaka (1939) reported that *Fimbristylis diphylloides* was $2n = 20$, while our results showed that $2n = 10$ for *F. diphylloides* (Table 1). This variation for *F. diphylloides* may be due to intraspecific polyploidy as intraspecific polyploidy ($2n = 10$ and 20) has been reported previously in this genus by Mehra & Sachdeva (1975) and Bir *et al.* (1988, 1992). The intraspecific polyploidy observed in this study therefore provides important evidence for polyploidization in the genus *Fimbristylis*.

Taxa with $2n = 16$ exhibited bimodal karyotypes, with 4 large chromosomes ranging from 3.9 μm to 4.4 μm and the other 12 chromosomes ranging from 1.9 μm to 2.8 μm in length (Fig. 4H). This karyotype has often been reported for the species of *Eleocharis* subseries *Eleocharis* (Strandhede 1965, Bureš *et al.* 2004, Yano *et al.* 2004). Based on karyotype analyses and molecular estimations of phylogeny in the genus *Eleocharis*, Yano *et al.* (2004) proposed that the $2n = 16$ condition results from chromosome fragmentation or fusion in species with $2n = 10$ or 20. In this study, taxa with $2n = 16$ that had bimodal karyotypes also appeared to have been derived from taxa with $x = 5$ by chromosome fragmentation or fusion. Those taxa with $2n = 24$ and bimodal karyotypes had 12 small chromosomes ranging in length from 0.9 μm to 1.3 μm and another 12 chromosomes ranging from 1.6 μm to 1.9 μm (Fig. 4K). In our phylogenetic tree (Fig. 3), the taxa with $2n = 24$ is closely related to the taxa with $2n = 10$, 20 or 30. Taxa with $2n = 24$ seemed to have been derived from the taxa with $2n = 10$, 20 or 30 by chromosome fragmentation or fusion, or they may have been hybrids with these taxa.

Hoshino (1981) reported that when the chromosome number increases by agmatoploidy (fragmentation) in the genus *Carex*, both the lengths and widths of all chromosomes tend to become smaller. Taxa with $2n = 42$, 44 and 52 had small chromosomes and appear to have been derived from the $2n = 10$ or 20 condition by fragmentation of diffuse centromeric chromosomes (Fig. 3). These data show that chromosomal evolution in the genus *Fimbristylis* may thus have been caused by both aneuploidization and polyploidization. This pattern of chromosomal evolution is similar to that in the genus *Eleocharis* (Yano *et al.* 2004). In order to further clarify the chromosomal evolution in the genus *Fimbristylis*, more tropical or subtropical species need to be included in future phylogenetic analyses.

The authors thank Hiroshi Ikeda, Machiko Tsusaka, Teruo Katsuyama, Tomomi Masaki, and Tomonori Hirahara for their great help in field trips and in collecting plant materials. We also thank Hiromi Tsubota and Hiroyuki Takasaki for advice about phylogenetic analyses.

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Received February 22, 2006; accepted August 11, 2006